Animal (2009), **3:1**, pp 1–5 © The Animal Consortium 2008
doi:10.1017/S1751731108003054

animal

Polymorphism identification, RH mapping and association of placental lactogen gene with milk production traits of dairy cows

J. Zhang[1], D. X. Sun[1], J. E. Womack[2], Y. C. Wang[1], Y. Yu[1], R. Liu[1] and Y. Zhang[1†]

[1]*Department of Animal Genetics and Breeding, Key Laboratory of Animal Genetics and Breeding of Ministry of Agriculture, China Agricultural University, Beijing 100094, China;* [2]*Department of Veterinary Pathobiology, Texas A&M University, College Station, TX 77843, USA*

(Received 13 January 2008; Accepted 3 June 2008; First published online 23 September 2008)

Bovine placental lactogen (bPL) is structurally related to prolactin (PRL) and growth hormone (GH). In synergism with steroid and thyroid hormones, bPL is crucial in stimulating the development of the mammary gland, mammary cell differentiation and function. To further explore whether bPL *gene is associated with milk production traits, we herein analyzed single-nucleotide polymorphisms (SNPs) within eight regions of* bPL *gene, which are potentially associated with five milk production traits on 1028 Chinese Holstein cows. Among these, two SNPs, NT7409(T–C) and Nt11246(G–A), were identified. The former is within exon 2; it induces an alteration of amino acid from Val to Ala. The later is within exon 4. It is a synonymous mutation. We found that there were significant associations between NT7409(T–C) and milk and protein yield. Cows of the AA genotype yielded less milk (*P = *0.001) and less protein (*P = *0.003) than those of genotypes AB and BB. However, on the NT11246(G–A) locus, no significant association was observed in the five milk production traits studied. In addition,* bPL *has been localized near markers* RM185 *and* CC549051 *with a distance of 23.2 cR on BTA 23. It is at the same position as the region including quantitative trait loci (QTLs) affecting milk and protein yields by previous linkage analysis. In summary, our findings demonstrated that the SNP within exon 2 of* bPL *(NT7409(T–C)) is associated with two milk production traits, and this provided further evidence that* bPL *could be a major gene-controlling milk production trait in Holstein dairy cattle.*

Keywords: *bPL*, association analysis, RH mapping, dairy cows

Introduction

Successful lactation in the dairy cow requires that the mammary glands produce a large number of potential milk-secreting cells during pregnancy and dry periods. Both mammary growth and initiation of milk synthesis are intimately dependent upon complex interactions among hypophyseal, adrenal, ovarian and placental hormones. Placental lactogens (PLs) are structurally related to prolactins (PRLs) and growth hormones (GHs), and play a crucial role in stimulating the development of the mammary gland in synergy with steroid and thyroid hormones (Cowie *et al.*, 1966; Schams *et al.*, 1984; Byatt *et al.*, 1994). Patel *et al.* (1996) reported that plasma bovine placental lactogen (bPL) levels were positively associated with the stage of gestation but not with fetal number, and the concentration of bPL in the fetus decreased with advancing gestation, whereas bPL concentration peaked in the maternal circulation during the last third of pregnancy and then reached a plateau. The gene coding bPL (*bPL*) spans approximately 12.5 kb, contains five exons, and encodes a predictive preprohormone of 236 amino acids with a signal peptide of 36 amino acids (Schuler *et al.*, 1988; Kessler and Schuler, 1991).

Based on the important roles in the growth and development of mammary gland (mammogenesis), synthesis of milk (lactogenesis), and maintenance of milk secretion (galactopoiesis), *bPL* is considered a strong candidate gene for milk production traits in dairy cattle. However, the associations between *bPL* and milk production traits, as well as physical location on the chromosome in dairy cattle remain undetermined. The objectives of the current study were to detect polymorphisms of *bPL* and determine association of such polymorphisms with milk production traits in Chinese Holstein cattle.

Material and methods

Animals

Blood samples of 1028 Chinese Holstein cows were collected randomly from eight Chinese Holstein cattle farms in

† E-mail: changy@cau.edu.cn

Beijing, including 12 sire families with 58 to 132 daughters from each sire. Regular and standard performance testing, i.e. DHI system, has been carried out in each farm since 1999. The individual phenotype for each of five milk production traits (i.e. milk yield, fat yield, protein yield, fat percentage and protein percentage over 305 days) is composed of two or three records corresponding to different parity, and were obtained from the Beijing Dairy Cattle Center. The total number of production records was 2324, and the mean and standard deviation (s.d.) of the five traits are presented in Table 1.

Genotyping and sequencing analysis

Genomic DNA was isolated from blood samples by the phenol–chloroform method. Using Oligo 6.0, eight pairs of primers corresponding to five exons and promotor regions were designed according to the genomic sequence of *bPL* (GenBank accession no. AH001151; Table 2). Polymerase chain reactions (PCR) were carried out in 20 μl volume including 50 ng of genomic DNA, 0.2 mM each primer and 0.5 U of Taq DNA polymerase. The PCR protocol was 94°C for 7 min, followed by 35 cycles of 94°C for 30 s, annealing for 45 s and 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR products were genotyped by single-strand conformation polymorphism (SSCP) to screen the mutations within the amplified region. In total, 2 μl of the PCR product of each sample was mixed with 8 μl of denaturing buffer (98% formamide, 0.09% xylene cyanole FF and 0.09% bromophenol blue) and then denatured at 98°C for 10 min, followed by a rapid chill on ice for 10 min. The denatured PCR products were electrophoresed on 12% polyacrylamide gels for 18 h at 8 V/cm and stained by 0.2% $AgNO_3$ for 20 min, and then 3% Na_2CO_3 for about 5 min (Qu *et al.*, 2005). Genotypes were recorded according to band patterns.

To identify the mutation site, the PCR products of homozygotes were purified using BioGene Geneclean III Kit (Carlsbad, CA, USA), and then sequenced on the ABI 377 DNA Sequencer using the BigDyeTM Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA). Both forward and reverse primers were applied to sequencing.

The PeptideStructure program of GCG version 11.1 (Accelrys Inc., San Diego, CA, USA) was used to predict the protein secondary structure.

Association analysis

Using an animal model and the additive genetic relationship matrix, the genotypic value, differences between genotypes, and additive and dominance effects for each trait were evaluated using the PEST software (Groeneveld, 1990), respectively. Pedigrees of animals detected in the present study were traced back to three generations to create the numerator relationship matrix, and hence the A^{-1} matrix included 2604 animals. The animal model is as follows:

$$y_i = hys_j + T_k + M_m + G_n + \alpha_i + p_i + e_i,$$

where y_i is the phenotypic value for each trait of cow i, hys_j the herd-year-season effect, T_k the parity number effect, M_m the month effect, G_n the fixed effect corresponding to the genotype of NT7409(T–C) or NT11246(G–A) (AA, BB, or AB), α_i the random polygenic component account for all known pedigree relationships ('animal model'; (Lynch and Walsh, 1997), including ungenotyped individuals whose phenotypes were ignored), p_i the permanent environmental

Table 1 *Means and s.d. of five milk production traits*

Traits[1]	Mean ± s.d.
Milk yield (kg)	8667.16 ± 1758.58
Fat yield (kg)	339.93 ± 73.74
Protein yield (kg)	283.12 ± 53.73
Fat percentage (%)	3.94 ± 0.52
Protein percentage (%)	3.28 ± 0.21

Table 2 *Primer sequences for promoter and exons in bPL*

Primers	Sequences	Tm (°C)	Amplicon
bPLpro1	F: 5′-GGAGAAGGGCATGATAAC-3′	59	Promoter
	R: 5′-GCAATAGGGAAAGATCAC-3′		−337 to −123
bPLpro2	F: 5′-TCCCTATTGCTCTTATGC-3′	56	Promoter
	R: 5′-ACTGAATGGAGGAAATC-3′		−132 to 35
bPL1	F: 5′-GTAGCACCCTATTTCTAT-3′	56	Exon 1
	R: 5′-TTTTGGAGAGTGAAGTAG-3′		(270 bp)
NT7409(T–C))	F: 5′-CAGGCTAACACATCATCT-3′	61	Exon 2
	R: 5′-ATCCCACTCACTTTCATC-3′		(290 bp)
bPL3	F: 5′-GCCAAAATAACCCAAAGG-3′	59	Exon 3
	R: 5′-TGTCTCAGTTGCTCAAGT-3′		(193 bp)
(NT11246(G–A))	F: 5′-TTACCAAGCCCACTGAAT-3′	56	Exon 4
	R: 5′-CTCACCCTTTTTTGTATC-3′		(244 bp)
bPL5	F: 5′-TTATCTTTGGGTGCTTAGGT-3′	59	Exon 5
	R: 5′-ATCATCACTAACCATCTCAG-3′		(244 bp)
bPL5a	F: 5′-GTCCTGAGATGGTTAGTG-3′	59	Exon 5a
	R: 5′-TCAGAGGTAGGGATGGAT-3′		(205 bp)

effect and e_i the random residual. All variance–covariance matrices have been estimated by restricted maximum likelihood using the EM algorithm, as applied in the REMLF90 programs (Misztal, 1999). Iterations were performed until the criterion of convergence was less than 1×10^{-7}.

The additive and dominance effects were estimated according to the following formulas (Falconer and Mackay, 1996):

$$a = (AA - BB)/2, \quad d = AB - (AA + BB)/2,$$

where *AA*, *BB* and *AB* are the genotypic values of homozygous and heterozygous genotypes.

Radiation hybrid (RH) mapping

RH mapping was carried out with a 5000-rad RH panel consisting of 90 cell lines and *bPL*-specific oligonucleotide, the forward and reverse primer sequences were 5′-ATCCATCCCGTCAAAAGC-3′ and 5′-CAGTTCCTGCTATTTGTG-3′ with an expected PCR product size of 230 bp (Womack *et al*., 1997). The PCR protocol was 94°C for 7 min, followed by 35 cycles of 94°C for 30 s, 54°C for 45 s, 72°C for 30 s and a final extension at 72°C for 10 min. The amplification of each cell line was assessed by electrophoresis in 2% agarose gel. The hybrids were scored for the presence/absence of the amplified product. Each marker was repeated twice to minimize possible experimental bias. Positions of the *bPL* on the map will be given relative to markers in the third-generation whole-genome comparative map of cattle and humans (Everts-van der Wind *et al*., 2005). RH analysis was carried out with RHMAPPER-1.22 (Slonim *et al*., 1997).

Results

RH mapping

We used RH to identify the genetic position of *bPL* on the chromosome. Statistical analysis of the PCR results on RH mapping vectors was carried out with RHMAPPER 1.22 (Slonim *et al*., 1997). Our results indicated that *bPL* is located near the markers *RM185* and *CC549051* on *Bos Taurus* autosome (BTA) 23 with distance 23.2 CR and LOD score 8.2.

Identification of polymorphisms

With eight pairs of primers, we successfully amplified out all the expected fragments for five exons and promoter region of *bPL*. After SSCP, two single-nucleotide polymorphisms (SNPs) were identified in exons 2 and 4. By comparing the sequences of two types of homozygotes on each SNP, we found that the SNP within exon 2 showed a nucleotide transition from T to C at +7409, called Nt7409(T–C), which led to an alteration of the amino acid from Val to Ala (Val 37Ala). On the other hand, the latter within exon 4 caused a base alteration of G into A at +11 246, named Nt11246(G–A). However, it was a synonymous mutation. As shown in Table 3, the frequency for Nt7409C (0.621) at the NT7409(T–C) locus was higher than that for Nt7409T (0.379), whereas for Nt11246(G–A), the frequency for Nt11246G (0.824) was much greater than that for Nt11246A (0.176).

Further, to determine whether NT7409(T–C) induces change of the protein structure, comparison of two kinds of protein secondary structure corresponding to Val 37 and Ala 37 was performed by the GCC 11.1 program. We found that one GOR turn was present at the position of Ala 37, but absent at the position of Val37, indicating NT7409(T–C) may change the dimensional structure and function of bPL (Figure 1).

Table 3 *Genotypic and allele frequencies of NT7409(T–C) and NT11246(G–A)*

NT7409(T–C)					NT11246(G–A)				
Genotype			Allele		Genotype			Allele	
TT	TC	CC	T	C	GG	GA	AA	G	A
0.10 ($n = 105$)	0.55 ($n = 554$)	0.35 ($n = 349$)	0.38	0.62	0.66 ($n = 679$)	0.33 ($n = 337$)	0.01 ($n = 12$)	0.82	0.18

Note: *n* means the number of cows.

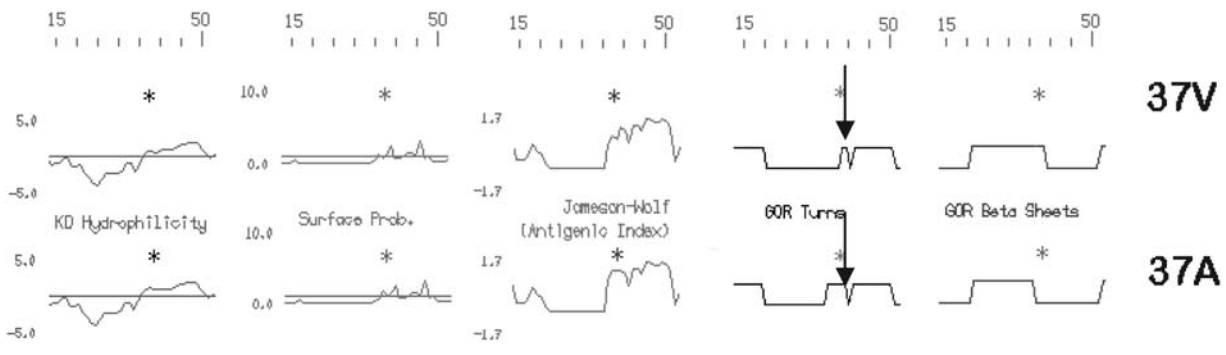

Figure 1 Prediction bPL protein secondary structure between Val 37 (37V) and Ala 37 (37A). The asterisk indicates position 37 and the arrowhead indicates GOR turn.

Association between bPL and milk production traits

With the PEST program, the genotypic values of milk yield, fat yield, protein yield, fat percentage and protein percentage for NT7409(T–C) and NT11246(G–A) were estimated, and the differences between different genotypic values of TT, CC or TC and additive and dominant effects of NT7409(T–C) and NT11246(G–A) loci for the five milk production traits were also tested. The cows with the TT genotype NT7409(T–C) had lower milk and protein yields than those with TC ($P = 0.005$ and 0.005; Table 4) and CC genotypes ($P = 0.001$ and 0.003; Table 4). Both milk and protein yields showed no significant dominance effects (Table 4). However, no significant association was observed between NT11246(G–A) and the five milk production traits (Table 5).

Discussion

Many previous studies have shown that bovine *bPL* plays an important role in stimulating the development of the mammary gland, differentiation and function of mammary cell to secrete milk and systemic adjustments in maternal metabolism in pregnancy and lactation (Cowie *et al*., 1966; Byatt *et al*., 1994; Patel *et al*., 1996). We, therefore, propose that a polymorphism in *bPL* may affect milk production traits in dairy cattle. In the present study, we first identified two polymorphic loci within exons 2 and 4 of *bPL*, of them, NT7409(T–C) within exon 2 led to a change of Val to Ala at position 37. Our association analysis further revealed that this SNP has significant associations with milk production traits. Interestingly, by comparing the secondary structure of bPL between Val 37 and Ala 37, we found that the mutation Val/Ala induced one GOR turn at position 37. Our findings implied that the missense mutation, NT7409(T–C), may be the principal factor for milk production traits by changing the dimensional confirmation and function of bPL. Determining the differences of the two profile types is critical to understanding the functional mechanism of the *bPL*. Further investigation is required to verify that the mutation found in this study affects the function of bPL, for example,

Table 4 *Comparison between different genotypic values, additive and dominance effects of NT7409(T–C) on the five milk production traits*

Traits[1]	Multiple comparison[2] ($\mu \pm$ s.e.)			Effect[3] ($\mu \pm$ s.e.)	
	TT–TC	TT–CC	TC–CC	Additive	Dominance
MY	-390.18 ± 138.72 ($P = 0.005$)**	-501.11 ± 155.35 ($P = 0.001$)**	-110.93 ± 101.87 ($P = 0.276$)	-250.56 ± 77.67 ($P = 0.001$)**	139.63 ± 93.69 ($P = 0.136$)
FY	-9.72 ± 5.54 ($P = 0.079$)	-11.05 ± 6.16 ($P = 0.073$)	-1.33 ± 4.04 ($P = 0.742$)	-5.53 ± 3.08 ($P = 0.073$)	4.20 ± 3.74 ($P = 0.262$)
PY	-11.77 ± 4.23 ($P = 0.005$)**	-13.89 ± 4.74 ($P = 0.003$)**	-2.12 ± 3.11 ($P = 0.495$)	-6.95 ± 2.37 ($P = 0.003$)**	4.82 ± 2.86 ($P = 0.092$)
FP	0.05 ± 0.04 ($P = 0.148$)	0.09 ± 0.04 ($P = 0.035$)	0.03 ± 0.03 ($P = 0.215$)	0.04 ± 0.02 ($P = 0.035$)	-0.01 ± 0.02 ($P = 0.690$)
PP	0.01 ± 0.02 ($P = 0.656$)	0.02 ± 0.02 ($P = 0.221$)	0.01 ± 0.01 ($P = 0.207$)	0.01 ± 0.01 ($P = 0.221$)	0.00 ± 0.01 ($P = 0.735$)

[1]MY = milk yield; FY = fat yield; PY = protein yield; FP = fat percentage; PP = protein percentage.
[2]Comparison between different genotypic values of MY, FY, PY, FP and PP for NT7409(T–C) locus.
[3]Additive and dominant effects of NT7409(T–C) locus for MY, FY, PY, FP and PP.

Table 5 *Comparison between different genotypic values, additive and dominance effects of NT11246(G–A) on the five milk production traits*

Traits[1]	Multiple comparison[2] ($\mu \pm$ s.e.)			Effect[3] ($\mu \pm$ s.e.)	
	GG–GA	GG–AA	GA–AA	Additive	Dominance
MY	72.21 ± 94.97 ($P = 0.447$)	-543.85 ± 382.01 ($P = 0.155$)	-616.05 ± 383.53 ($P = 0.108$)	-271.92 ± 191.01 ($P = 0.155$)	-344.13 ± 203.90 ($P = 0.092$)
FY	-0.69 ± 3.77 ($P = 0.854$)	-32.528 ± 15.14 ($P = 0.032$)	-31.84 ± 15.20 ($P = 0.036$)	-16.26 ± 7.57 ($P = 0.032$)	-15.57 ± 8.08 ($P = 0.054$)
PY	0.73 ± 2.89 ($P = 0.801$)	-19.28 ± 11.63 ($P = 0.098$)	-20.01 ± 11.68 ($P = 0.087$)	-9.639 ± 5.818 ($P = 0.098$)	-10.37 ± 6.21 ($P = 0.095$)
FP	-0.04 ± 0.03 ($P = 0.170$)	0.13 ± 0.10 ($P = 0.195$)	-0.10 ± 0.10 ($P = 0.343$)	-0.065 ± 0.050 ($P = 0.195$)	-0.03 ± 0.05 ($P = 0.568$)
PP	-0.02 ± 0.01 ($P = 0.131$)	-0.01 ± 0.04 ($P = 0.801$)	0.01 ± 0.04 ($P = 0.903$)	-0.01 ± 0.02 ($P = 0.801$)	0.01 ± 0.02 ($P = 0.641$)

[1]MY = milk yield; FY = fat yield; PY = protein yield; FP = fat percentage; PP = protein percentage.
[2]Comparison between different genotypic values of MY, FY, PY, FP and PP for NT11246(G–A) locus.
[3]Additive and dominant effects of NT11246(G–A) locus for MY, FY, PY, FP and PP.

determination of differences in the protein expression of different genotypes.

So far, in dairy cattle, many quantitative trait loci (QTLs) affecting milk production traits have been identified (http://www.vetsci.usyd.edu.au/reprogen/QTL_Map/), of which BTA3, 6, 9, 14, 20 and 23 were shown to harbor QTLs with pleiotropic effect on multiple milk production traits (Khatkar *et al*., 2004). Bennewitz *et al*. (2003) reported that on BTA23 existed a QTL affecting milk and protein yields at a position around 60 cM. In this study, *bPL* was mapped to be close to *RM185* by RH mapping, which was located near 60 cM on BTA 23 (Naoya *et al*., 2004). Our association result is consistent with that of linkage analysis by Bennewitz *et al*. (2003), implying that *bPL* may be a QTL on BTA23 affecting milk production traits. However, much more in-depth analysis will be required for the chromosomal region around 60 cM and the protein-folding work of NT7409(T–C) to prove whether the mutation is the casual site.

Taken together, our association analysis revealed an SNP within exon 2 of *bPL*, which showed significant associations with four milk production traits in Chinese Holstein. Further, *bPL* has been localized near *RM185* on BTA 23 and at the same position as the region identified by previous linkage mapping. Based on the findings of this study, we think that *bPL* could be a potential useful genetic marker in a selection program in Holstein cattle.

Acknowledgments

This work was supported by the '973' Project (2006CB102107), '948' Project (2006-G48), '863' Project (2007AA10Z157) and the National Key Technologies R&D Program (2006BAD04A01).

References

Bennewitz J, Reinsch N, Grohs C, Leveziel H, Malafosse A, Thomsen H, Xu N, Looft C, Kuhn C, Brockmann GA, Schwerin M, Weimann C, Hiendleder S, Erhardt G, Medjugorac I, Russ I, Forster M, Brenig B, Reinhardt F, Reents R, Averdunk G, Blumel J, Boichard D and Kalm E 2003. Combined analysis of data from two granddaughter designs: a simple strategy for QTL confirmation and increasing experimental power in dairy cattle. Genetics Selection Evolution 35, 319–338.

Byatt JC, Eppard PJ, Veenhuizen JJ, Curran TL, Curran DF, McGrath MF and Collier RJ 1994. Stimulation of mammogenesis and lactogenesis by recombinant bovine placental lactogen in steroid-primed dairy heifers. Journal of Endocrinology 140, 33–43.

Cowie AT, Tindal JS and Yokoyama A 1966. The induction of mammary growth in the hypophysectomized goat. Journal of Endocrinology 34, 185–195.

Falconer DS and Mackay TFC 1996. Introduction to quantitative genetics, 4th edition. Longman Scientific and Technical, New York.

Groeneveld E 1990. PEST user's manual. Institute of Animal Husbandry and Animal Behaviour Federal Agricultural Research Centre (FAL), Mariensee, Germany.

Kessler MA and Schuler LA 1991. Structure of the bovine placental lactogen gene and alternative splicing of transcripts. DNA and Cell Biology 10, 93–104.

Khatkar MS, Thomson PC, Tammen I and Raadsma HW 2004. Quantitative trait loci mapping in dairy cattle: review and meta-analysis. Genetics Selection Evolution 36, 163–190.

Lynch M and Walsh B 1997. Genetics and analysis of quantitative traits. Sinauer Associates, Inc., Sunderland, MA, USA.

Misztal I 1999. Complex models, more data: simpler programming. Proceedings of the International Workshop on Computerized Cattle Breeding '99, Bulletin 20, Interbull, Tuusala, Finland.

Naoya I, Takasuga A, Mizoshita K, Takeda H, Sugimoto M, Mizoguchi Y, Hirano T, Itoh T, Watanabe T, Reed KM, Snelling WM, Kappes SM, Beattie CW, Bennett GL and Sugimoto Y 2004. A comprehensive genetic map of the cattle genome based on 3802 microsatellites. Genome Research 14, 1987–1998.

Patel OV, Hirako M, Takahashi T, Sasaki N and Domeki I 1996. Plasma bovine placental lactogen concentration throughout pregnancy in the cow. Relationship to stage of pregnancy, fetal mass, number and postpartum milk yield. Domestic Animal Endocrinology 13, 351–359.

Qu LJ, Li XY, Wu GQ and Yang N 2005. Efficient and sensitive method of DNA silver staining in polyacrylamide gel. Electrophoresis 26, 99–101.

Schams D, Russe I, Schellenberg E, Prokopp S and Chan JSC 1984. The role of steroid hormones, prolactin and placental lactogen on mammary gland development in ewes and heifers. Journal of Endocrinology 102, 121–130.

Schuler LA, Shimomura K, Kessler MA, Zieler CG and Bremel RD 1988. Bovine placental lactogen: molecular cloning and protein structure. Biochemistry 27, 8443–8448.

Slonim D, Kruglyak L, Stein L and Lander E 1997. Building human genome maps with radiation hybrids. Journal of Computational Biology 4, 487–504.

Everts-van der Wind A, Larkin DM, Green CA, Elliott JS, Olmstead CA, Chiu R, Schein JE, Marra MA, Womack JE and Lewin HA 2005. A high-resolution whole-genome cattle–human comparative map reveals details of mammalian chromosome evolution. Proceedings of the National Academy of Sciences 102, 18526–18531.

Womack JE, Johnson JS, Owens EK, Rexroad CE, Schlapfer J and Yang YP 1997. A whole-genome radiation hybrid panel for bovine gene mapping. Mammalian Genome 8, 854–856.